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Genotyping of *Toxoplasma gondii* isolates from chickens from India

C. Sreekumar [a,*], D.H. Graham [b], E. Dahl [b], T. Lehmann [b], M. Raman [c], D.P. Bhalerao [d], M.C.B. Vianna [a], J.P. Dubey [a]

[a] *United States Department of Agriculture, Animal Parasitic Diseases Laboratory, Animal and Natural Resources Institute, Agricultural Research Service, Beltsville, MD 20705-2350, USA*
[b] *Division of Parasitic Diseases, Centers for Disease Control and Prevention, 4770 Buford Highway, MS: F22, Chamblee, GA 30341, USA*
[c] *Department of Parasitology, Veterinary College and Research Institute, Namakkal, Tamil Nadu 637 001, India*
[d] *Department of Medicine, Bombay Veterinary College, Parel, Mumbai 400 012, India*

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Abstract

The present study was undertaken to isolate and genotype *Toxoplasma gondii* from free-range chickens (*Gallus domesticus*) from villages in Maharashtra and Tamil Nadu states of central and south India, respectively. Blood, heart, and brain from a total of 741 chickens were examined for *T. gondii* infection. Antibodies to *T. gondii*, as assayed with the modified agglutination test (MAT $\geq$ 1:5) were found in 133 (17.9%) chickens. Hearts and brains of 186 chickens were bioassayed in mice. Additionally, hearts and/or brains of most of the seronegative (MAT $<$ 1:5) chickens were fed to 20 *T. gondii*-free cats, while 32 seropositive chickens (MAT 1:5) were fed to 3 cats. *T. gondii* was not isolated from any of the chickens by mouse bioassay. Five of the cats that were fed seronegative chickens shed oocysts, while isolates were not obtained from any of the other cats fed seropositive chickens. These five isolates, along with the two that were previously isolated in India through cat bioassay, were genetically analyzed. Genotyping using the SAG 2 locus indicated that two isolates were type II and five were type III. Microsatellite analysis revealed allelic differences between and within the lineages. This is the first report of genetic characterization of any *T. gondii* isolate from India.
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Keywords: Toxoplasma gondii; Chicken; *Gallus domesticus*; India; Isolation; Genotyping; Microsatellite analysis

* Corresponding author. Tel.: +1-301-504-5999/8984; fax: +1-301-504-9222.
E-mail address: kumar@anri.barc.usda.gov (C. Sreekumar).

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1. Introduction

Infections by the protozoan *Toxoplasma gondii* are widely prevalent in humans and animals causing significant impact on animal production and public health throughout the world (Dubey and Beattie, 1988). Food-borne transmission of *T. gondii* is increasingly recognized as potentially a more important source of infection to humans than cats in many endemic areas. Another common mode of infection is by ingestion of food and drink contaminated with sporulated oocysts. The prevalence of *T. gondii* in free-range chickens is a good indicator of the prevalence of *T. gondii* oocysts in the environment, due to their habits of scratching the earth and feeding, facilitating greater access to the hidden feces of cats.

Genetic variation in *T. gondii* is low, and isolates have been classified into three genetic types (I–III) based on restriction fragment length polymorphism (RFLP) (Howe and Sibley, 1995; Howe et al., 1997). Numerous studies have shown that most isolates obtained from animals belong to types II and III (Howe and Sibley, 1995; Mondragon et al., 1998; Owen and Trees, 1999; Jungersen et al., 2002). Recently, a higher proportion of isolates of *T. gondii* from asymptomatic free-range chickens from rural areas surrounding São Paulo, Brazil were type I (Dubey et al., 2002). In contrast most of the isolates from rural Egypt were found to be of type III (Dubey et al., 2003a). Little is known of the biological and molecular characters of isolates of *T. gondii* from animals or humans from India. This is the first report of genetic characterization of *T. gondii* isolates from India.

2. Materials and methods

2.1. Naturally infected chickens

Native, free-range chickens were obtained from two Indian states of Tamil Nadu (south India) and Maharashtra (central India) (Table 1). The chickens were collected by the dealers from numerous villages and slaughtered at a destined place for sale. The hearts and the corresponding sera samples were separately collected in marked containers. On occasions, the entire head was also collected. The samples were transported, so as to reach USDA's laboratory in Beltsville, in 7 days. During this time the samples had been stored at room temperature (22–30 °C). A total of 10 batches were received, three from Maharashtra and seven from Tamil Nadu (Table 1).

2.2. Serologic examination

Sera from chickens were diluted twofold starting at 1:5 or 1:10 dilution and assayed for *T. gondii* antibodies with the modified agglutination test (MAT) as described (Dubey and Desmonts, 1987).

2.3. Bioassay of chicken tissues in mice

The procedures were identical to those described by Dubey et al. (2002). Brains and hearts of seropositive (MAT 1:5 or more) chickens were bioassayed individually in groups of mice

Table 1
Prevalence of *T. gondii* among free-range chickens from India

City/date received	Number of samples received	Number seropositive (number with titers)	Isolates obtained by passage		
			Mouse[a]	Cat	
				Number of chicken tissues fed[b]	Oocyst shedding (isolate designation)
Chennai/30 November 2001	52	4 (1-1 in 10, 3-1 in 80)	0/4	6	0/1
				10	1/1 (TgCInd-3)[c]
				10	0/1
				11	0/1
				11	0/1
Chennai/17 January 2002	45	0	ND[d]	13	1/1 (TgCInd-4)
Namakkal[e]/30 May 2002	68	20 (7-1 in 5, 10-1 in 10, 2-1 in 20, 1-1 in 40)	0/17	25	1/1(TgCInd-5)
				23	1/1 (TgCInd-6)
Namakkal[e]/21 June 2002	109	2[f] (1 in 10)	0/108[g]	ND	
Mumbai/22 July 2002	50	12 (6-1 in 5, 3-1 in 10, 3-1 in 20)	0/11	20	0/1
				18	1/1 (TgCInd-7)
Namakkal[e]/25 July 2002	115	15 (5-1 in 5, 8-1 in 10, 1-1 in 20, 1-1 in 40)	0/10	26	0/1
				29	0/1
Mumbai/6 August 2002	50	24 (13-1 in 5, 11-1 in 25)	0/11	14	0/1
				19	0/1
				12	0/1
Namakkal[e]/23 August 2002	101	22 (21-1 in 5, 1-1 in 25)	0/11	33	0/1
				22	0/1
				18	0/1

Table 1 (*Continued*)

City/date received	Number of samples received	Number seropositive (number with titers)	Isolates obtained by passage		
			Mouse[a]	Cat	
				Number of chicken tissues fed[b]	Oocyst shedding (isolate designation)
Chennai[h] and Namakkal[e]/ 19 September 2002	81	20 (1 in 5)	ND	10	0/1
				8	0/1
				15[i]	0/1
				31	0/1
				29	0/1
Mumbai/28 October 2002	70	14 (1 in 5)	0/14	ND	
Total	741	133	0/186	5/23	

[a] Number of groups positive/number of groups infected.
[b] Fed chickens with titers below 1:10.
[c] *Toxoplasma gondii* chicken, India. The two isolates brought as oocysts from India were designated as TgCInd-1 and -2.
[d] Not done.
[e] Chickens collected form small villages and towns from the districts of Namakkal, Salem and Erode, an area of around 50 km radius.
[f] Serum obtained from heart.
[g] All available samples bioassayed in mice as sera samples were not available in this batch.
[h] Chickens collected from villages in Chennai and Chingleput districts, an area of around 30 km radius.
[i] No sera available.

after digestion in pepsin. For this, brain and heart of each chicken were pooled, homogenized in five volumes (w/v) of aqueous 0.85% NaCl (saline), mixed with five volumes of acidic pepsin, the mixture incubated in a shaker water bath for 1 h at 37 °C, centrifuged, neutralized, mixed with antibiotics (Dubey, 1998), and the homogenate was inoculated subcutaneously (s.c.) into five mice. The mice used were Swiss Webster albino females obtained from Taconic Farms, German Town, New York. Tissue imprints of mice that died were examined for *T. gondii* tachyzoites or tissue cysts. Survivors were bled on day 45 post-inoculation (p.i.), and a 1:25 dilution of serum from each mouse was tested for *T. gondii* antibodies by MAT. Mice were killed 50 days p.i. and their brains were examined microscopically for tissue cysts.

2.4. Bioassay of chicken tissues in cats

Hearts and brains from chickens with MAT titers of less than 1:10 were pooled and fed to cats (Table 1). The cats fed with chicken tissues were monitored for infection by examination of their feces. Oocysts were collected from cats using Sheather's sugar solution and allowed to sporulate in 2% sulfuric acid (Dubey and Beattie, 1988).

2.5. Oocyst titration

The sporulated oocysts were counted and titrated for infectivity by feeding mice with 10-fold dilutions and noting the last dilution that resulted in sero-conversion. The identity of the oocysts was confirmed as *T. gondii* by two subsequent passages in mice.

Oocysts from the two isolates that were previously obtained from chickens from the Izatnagar and Chennai regions of India (Sreekumar et al., 2001) were also passaged in mice to confirm their identity as *T. gondii*. These isolates had been obtained by feeding chicken hearts to 8–12-week-old cats in India. The oocysts had been stored for 22 months before they were brought to the Beltsville laboratory. The oocysts of the Izatnagar isolate were titrated by feeding 10-fold serial dilution to mice. Brain squash preparations, from the mice of the seropositive groups, were examined for the presence of tissue cysts. Positive tissues from the mice fed the highest dilution were used for DNA extraction. Oocysts of the Chennai isolate were fed to mice and tissues of a mouse that was infected was subpassaged. DNA was extracted from the brains of infected mice.

2.6. Genotyping and microsatellite analysis of T. gondii isolates

Frozen extracts of lung, mesenteric lymph node or brain were used for obtaining *T. gondii* DNA (Lehmann et al., 2000). The clonal lineages were determined by restriction fragment length polymorphisms of the SAG2 gene (Howe et al., 1997). Microsatellite genotypes were scored as per the procedures outlined by Blackston et al. (2001).

3. Results

A total of 571 samples from Tamil Nadu and 170 samples from Mumbai were examined for *T. gondii*. Antibodies to *T. gondii* (1:5 and above) were found in 133 (17.9%) of 741

Table 2
Genotypes of *T. gondii* isolates from chickens from India

Serial number	Isolate designation	Genotype at SAG2 locus	Microsatellite locus					
			M6	M33	M48	M102	M163	M95
1	TgCInd-1	III	200	167	213	191	174	402
2	TgCInd-2	III	200	167	215	191	174	402
3	TgCInd-3	II	200	167	217	175	172	228
4	TgCInd-4	III	200	167	215	191	176	402
5	TgCInd-5	II	216	171	215	175	164	221
6	TgCInd-6	III	200	167	215	191	178	402
7	TgCInd-7	III	200	167	215	191	174	ND[a]

[a] Not done.

chickens. The MAT titers of chickens were 1:5 in 88 chickens, 1:10 in 22 chickens, 1:20 in 6 chickens, 1:25 in 12 chickens 1:40 in 2 chickens and 1:80 in 3 chickens (Table 1).

T. gondii oocysts were obtained from five of the 23 cats fed tissues of chickens. The identity of all the five isolates obtained in the present study and the two isolated by Sreekumar et al. (2001) was established as *T. gondii* by two consecutive passages in mice. Tissue cysts were found in the brains of mice infected with these isolates. None of these isolates were pathogenic to mice.

Of the seven isolates genotyped, two were type II and five were type III (Table 2). Microsatellite analysis revealed that types II and III genotypes could further be characterized into two and three distinct multi-locus genotypes, respectively (Table 2).

T. gondii was not isolated from tissues of any of the seropositive chickens by bioassay in mice. All the 186 groups of mice injected with the pepsin digest of the seropositive chicken hearts remained seronegative for antibodies to *T. gondii*. Tissue cysts were not seen in the mice when they were euthanized 8 weeks after inoculation with chicken tissues.

4. Discussion

In the present study, a prevalence rate of 17.9% was recorded using MAT. Earlier reports have indicated prevalence rate of 39.5% (Devada et al., 1998) using MAT and 21.7% using an indirect fluorescent antibody test (Sreekumar et al., 2001) among free-range chicken in India. The lower prevalence rate in the present study even at a cut of level of 1:5 could be due to various reasons. It is possible that the samples that were analyzed herein originated from younger birds. This theory is supported by the fact that the highest titer obtained in this study was only 1:80, which was much less than that obtained elsewhere (Dubey et al., 2002, 2003a). Thus it is entirely possible that a younger age group of birds were sampled leading to a lower prevalence. Storage of sera at room temperature might have also affected the antibody levels.

In the present study, sera were assayed by MAT starting at a lower dilution (1:5) compared with 1:25 dilution used previously (Devada et al., 1998) because occasionally *T. gondii* has been isolated from chickens with antibodies lower than 1:25 (Dubey et al., 2002). *T. gondii*

was isolated from the feces of five cats fed tissues pooled from 89 chickens with titers of 1:5 or less. The lack of isolation of *T. gondii* from the 288 seronegative (MAT < 1:5) chickens fed to 20 cats suggests that the incidence of false negative results with MAT is low.

T. gondii could not be isolated from any of the positive samples by bioassay in mice. Though MAT is considered to be a fairly consistent indicator of the seroprevalence in chickens (Dubey et al., 1993) the threshold MAT titer indicative of *T. gondii* infection in chickens has not been determined. While successful isolation was possible from the chicken with titer below 1:20 from Argentina (Dubey et al., 2003c) isolation was not possible from chicken with titer of 1:80 in the present study. It is likely that storage of tissues of under unrefrigerated conditions during transport from India to USA affected the viability of *T. gondii*.

In the present study, types II and III genotypes were isolated form Indian chickens. Similar results have been obtained form chicken from free-range chickens from the United States (Dubey et al., 2003a). While a considerable proportion of the isolates from Brazil have been of type I (Dubey et al., 2002, 2003b), no type I isolate was obtained in the present study. Although a sample size of 7 is too small for definite conclusions, the lineage composition (based on SAG2) of the Indian chicken appears more similar to that of Egypt and the USA than that of Brazil. Microsatellite analysis showed five distinct multi-locus genotypes among the seven isolates, suggesting considerable genetic diversity of *T. gondii* in India.

References

Blackston, C.R., Dubey, J.P., Dotson, E., Su, C., Thulliez, P., Sibley, D., Lehmann, T., 2001. High-resolution typing of *Toxoplasma gondii* using microsatellite loci. J. Parasitol. 87, 1472–1475.

Devada, K., Anandan, R., Dubey, J.P., 1998. Serologic prevalence of *Toxoplasma gondii* in chickens in Madras, India. J. Parasitol. 84, 621–622.

Dubey, J.P., 1998. Refinement of pepsin digestion method for isolation of *Toxoplasma gondii* from infected tissues. Vet. Parasitol. 74, 75–77.

Dubey, J.P., Beattie, C.P., 1988. Toxoplasmosis of Animals and Man. CRC Press, Boca Raton, FL, p. 220.

Dubey, J.P., Desmonts, G., 1987. Serological responses of equids fed *Toxoplasma gondii* oocysts. Equine Vet. J. 19, 337–339.

Dubey, J.P., Ruff, M.D., Camargo, M.E., Shen, S.K., Wilkins, G.L., Kwok, O.C.H., Thulliez, P., 1993. Serologic and parasitologic responses of domestic chickens after oral inoculation with *Toxoplasma gondii* oocysts. Am. J. Vet. Res. 54, 1668–1672.

Dubey, J.P., Graham, D.H., Blackston, C.R., Lehmann, T., Gennari, S.M., Ragozo, A.M.A., Nishi, S.M., Shen, S.K., Kwok, O.C.H., Hill, D.E., Thulliez, P., 2002. Biological and genetic characterisation of *Toxoplasma gondii* isolates from chickens (*Gallus domesticus*) from São Paulo, Brazil: unexpected findings. Int. J. Parasitol. 32, 99–105.

Dubey, J.P., Graham, D.H., Dahl, E., Hilali, M., El-Ghaysh, A., Sreekumar, C., Kwok, O.C., Shen, S.K., Lehmann, T., 2003a. Isolation and molecular characterization of *Toxoplasma gondii* from chickens and ducks from Egypt. Vet. Parasitol. 114, 89–95.

Dubey, J.P., Navarro, I.T., Graham, D.H., Dahl, E., Freire, R.L., Prudencio, L.B., Sreekumar, C., Vianna, M.C., Lehmann, T., 2003b. Characterization of *Toxoplasma gondii* isolates from free range chickens from Paraná. Brazil. Vet. Parasitol. 117, 229–234.

Dubey, J.P., Venturini, M.C., Venturini, L., Piscopo, M., Graham, D.H., Dahl, E., Sreekumar, C., Vianna, M.C., Lehmann, T., 2003c. Isolation and genotyping of *Toxoplasma gondii* from free ranging chickens from Argentina. J. Parasitol. 89, 1063–1064.

Howe, D.K., Sibley, L.D., 1995. *Toxoplasma gondii* comprises three clonal lineages: correlation of parasite genotype with human disease. J. Infect. Dis. 172, 1561–1566.

Howe, D.K., Honoré, S., Derouin, F., Sibley, L.D., 1997. Determination of genotypes of *Toxoplasma gondii* strains isolated from patients with toxoplasmosis. J. Clin. Microbiol. 35, 1411–1414.

Jungersen, G., Jensen, L., Rask, M.R., Lind, P., 2002. Non-lethal infection parameters in mice separate sheep Type II *Toxoplasma gondii* isolates by virulence. Comp. Immunol. Microbiol. Infect. Dis. 25, 187–195.

Lehmann, T., Blackston, C.R., Parmley, S.F., Remington, J.S., Dubey, J.P., 2000. Strain typing of *Toxoplasma gondii*: comparison of antigen-coding and house-keeping genes. J. Parasitol. 86, 960–971.

Mondragon, R., Howe, D.K., Dubey, J.P., Sibley, L.D., 1998. Genotypic analysis of *Toxoplasma gondii* isolates from pigs. J. Parasitol. 84, 639–641.

Owen, M.R., Trees, A.J., 1999. Genotyping of *Toxoplasma gondii* associated with abortion in sheep. J. Parasitol. 85, 382–384.

Sreekumar, C., Rao, J.R., Mishra, A.K., Ray, D., Singh, R.K., Joshi, P., 2001. First isolation of *Toxoplasma gondii* from chicken in India. J. Vet. Parasitol. 15, 103–106.